Katiyar et al | Article 8604
J Pure Appl Microbiol. 2023;17(3):1669-1678. doi: 10.22207/JPAM.17.3.30
Received: 05 April 2023 | Accepted: 19 June 2023
Published Online: 01 September 2023

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Concentration Dependent Effect of Azadirachta indica (Neem) Seed Oil and Neem Bark extract on Planktonic and Established Biofilm Growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Ravish Katiyar[1], Ekta Khare[2] and Shilpa Deshpande Kaistha[2,3]*

[1]Department of Microbiology, Dr. Shakuntala Misra National Rehabilitation University, Lucknow, Uttar Pradesh, India.
[2]Department of Life Sciences & Biotechnology, School of Life Sciences & Biotechnology, Chhatrapati Shahu Ji Maharaj University, Kanpur, Uttar Pradesh, India.
[3]Department of Biotechnology, School of Life Sciences & Biotechnology, Chhatrapati Shahu Ji Maharaj University, Kanpur, Uttar Pradesh, India.

***Correspondence:** shilpakaistha@csjmu.ac.in

Citation: Katiyar R, Khare E, Kaistha SD. Concentration Dependent Effect of Azadirachta indica (Neem) Seed Oil and Neem Bark extract on Planktonic and Established Biofilm Growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus. J Pure Appl Microbiol.* 2023;17(3):1669-1678. doi: 10.22207/JPAM.17.3.30

Abstract

***Azadirachta indica* Juss (Neem) is well documented for its antimicrobial activity. The effect of varying concentrations (0.1 to 50% v/v) of *Azadirachta indica* derived neem seed oil (NSO), neem seed oil with tween 20 and neem bark extract was evaluated on planktonic, biofilm formation and mature biofilms of multiple drug resistant *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 25923 using the crystal violet assay and scanning electron microscopy. NSO showed antimicrobial activity at 25% v/v for *P. aeruginosa* but not *S. aureus* in zone of inhibition assay. Neem bark extract on the contrary showed antimicrobial activity against both the isolates at 50% v/v concentrations. Interestingly, in biofilm formation assay, low concentrations of NSO (3.5 to 0.2% v/v) induced biofilm formation while inhibition of both planktonic and biofilm was seen in concentration dependent manner from 12.5% v/v onwards. Complex of NSO and tween in comparison of NSO alone caused low induction in *S.aureus* biofilm formation, while inhibiting biofilm formation of *P. aeruginosa* at all the concentrations. In biofilm eradication assay, NSO induced biofilm of both *P. aeruginosa* (50 to 0.1%v/v) and *S. aureus* (50 to 3.13%v/v). Eradication effect of neem bark extract was found on *P. aeruginosa* biofilm in a dose dependent fashion from 50 to 20% v/v followed by 0.2 to 0.1%v/v concentration respectively. *S. aureus* biofilm were eradicated at 50 to 25%v/v concentrations. At low concentrations, both the neem derivatives induced biofilm mediated growth of the pathogenic organisms. The data also indicate that neem seed oil was more effective against Gram negative *P. aeruginosa* while neem bark extract was effective against Gram positive *S. aureus.* This study highlights the crucial but variable effects of concentration dependent effect of phytochemicals and their composition on biofilm induction as well as eradication, the primary growth form in clinical settings. This challenges the notion that all herbal products are safe as antimicrobial activities differ as per microbial growth modes. Hence, concentration dependent effect of medicinal plant derived products requires thorough investigation prior to their use as antimicrobial agents.**

Keywords: Neem Seed Oil, Neem Bark, *Pseudomonas aeruginosa, Staphylococcus aureus,* Biofilm

INTRODUCTION

Azadirachta indica A. Juss. (popularly known as Neem), a member of the Meliceaea family is a common tropical tree, described for its many antimicrobial, anthelmintic, antiemetic, antacid, antileprotic, antipyretic, analgesic, mosquito repellant, antifertility activities.[1] Neem limonoid extracts belonging to nine different structural groups azadirone (from oil), amoorastatin (from fresh leaves), vepinin (from seed oil), vilasinin (from green leaves), gedunins (from seed oil and bark), neem bark extract (from leaves and seed), nimbolin (from kernels), salanin (from fresh leaves and seed) and azadirachtin (from neem seed) have been described.[1] Neem seed oil and leaf extract has been described as the most medicinal part of the tree.[2,3] The bitter seed oil has been traditional used for the treatment of skin and gastric ulcers, malarial parasites and as an antiviral agent.[2] Neem seed extracts also exhibit anti-biofilm and anti-cancer activities.[4,5] Neem bark also contains many phenolics and tannin like substances that inhibits bacterial growth.[6]

Pseudomonas aeruginosa, gram negative bacilli causative agent of nosocomial infections is a potent biofilm former which is implicated in a number of nosocomial infections.[7] *Staphylococcus aureus*, a gram positive cocci also contributes to pathogenic infections such as damaged skin infections, ocular infections, chronic and recurrent airway infections, osteomyelitis and biofilm infections in food industry.[8] Biofilms are community driven microbial growth forms typically adhering on abiotic or biotic substrates encompassed by the secreted biopolymeric matrix that protects the residents from environmental stresses including antimicrobial compounds.[9] The antibacterial activity of neem seed oil and neem bark extract has been demonstrated for a number of micro-organisms,[10] but its effect implicitly on biofilm formation and eradication and biofilm dispersal are recently being investigated on *Pseudomonas aeruginosa* and *Staphylococcus*

aureus.[4,11,12] Bacteria within biofilms are several fold more resistant to antimicrobial agents and chemotherapeutic agents.[13] In the recent development of antibiotic resistance and the inclusion of these pathogens in the antibiotic surveillance list of ESKAPE (*Enterococcus caesum, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacter sp.*) pathogen, it is imperative that alternative means of pathogen control be investigated. Here we studied the concentration dependent effect of neem seed oil and neem bark extract on *P. aeruginosa* and *S. aureus* biofilm forming multiple drug resistant strains in their planktonic and biofilm growth forms. To the best of our knowledge there is no report of concentration based study of neem extract on planktonic, biofilm formation and biofilm eradication of *P. aeruginosa* and *S. aureus*. The outcome of the study is determination of the effective concentration for the antibiofilm/antimicrobial activity of Neem based extracts for antibiotic resistant pathogens.

MATERIALS AND METHODS

Cultures and Chemicals

Pseudomonas aeruginosa ATCC 15422 and *Staphylococcus aureus* ATCC 25923 were used as test organisms. The isolates were grown on trypticase soyapeptone media at 37°C for 24h. Analytical grade Need seed oil (NSO) obtained from HiMedia (India) was used directly and in the presence of 10% Tween 20 solutions diluted in trypticase soyapeptone media. Methanolic neem bark extract (NBE) was prepared as per protocol.[14] 100 mg/ml methanolic NBE was diluted in sterile saline to prepare varying concentrations. Media were procured from HiMedia, India and analytical grade chemicals from Merck India.

Agar Diffusion Assay

Antimicrobial activity was also determined by measuring zone of inhibition in mm post incubation using Agar diffusion assay.[15] The log phase bacterial culture was spread on the Mueller-Hinton agar (Hi Media, India). A sterile well cutter was used to punch the agar and varying concentration of NSO, NSO Tween and NBE. The plates were incubated at 37°C for 24 hours. The antibacterial activity was evaluated by measuring the zone of inhibition. The experiments were carried out in triplicates.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of NSO, NSO Tween and NBE were determined in varying concentrations (0.1 to 50% v/v) in tryptic soy broth using broth micro dilution method (2 fold dilution) in polystyrene micro titer plates.[16,17] MIC was defined as the lowest concentration without visible growth. MBC was defined as the lowest concentration reducing inoculums by > 99%.

Static Biofilm formation Assay and cell viability

Effect of NSO, NSO Tween and NBE was determined on biofilm formation according to the modified protocol.[17] Briefly, different concentrations (0.3-50 v/v %) were dispensed in microtitier plates containing log phase cultures or 24h preformed biofilms. After incubation at 37°C for 24h, each well was washed, dried and stained with 1% crystal violet for 20 minutes. Stained biofilm was washed to remove excess crystal violet and resuspended in DMSO and OD measured using micro titer ELISA plate reader.

Scanning Electron Microscopy

The architecture of biofilm in presence and absence of neem seed oil were analyzed using scanning electron microscopy. Briefly, coverslips were placed in media containing different concentrations of neem bark and neem seed oil and incubated for 24h at 37°C.[17] Biofilms were initially fixed in 2% glutaraldehyde for 30 min. Fixed cells were washed with 1 x PBS, and then serially dehydrated in upgrade ethanol (30%, 50%, 70%, 90% and 100%) for 15 to 20 min each. Samples were coated with gold in the sputtering machine and examined using scanning electron microscope (Zeiss, Advanced Instrumentation Research Facility, JNU, New Delhi.

Statistical Analysis

Statistical analysis was done with a Student's paired t-test. P value ≤ 0.05 was considered biological significant.

RESULTS

Effect of Neem Seed Oil and Neem Bark Extract on microbial growth

The antimicrobial activity of different concentrations of neem seed oil (NSO) and neem bark extract (NBE) were tested for *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 259836 using Agar diffusion assay.[15] Accordingly, Figure 1 shows that while 50% v/v concentration of NSO shows antimicrobial activity against only *Pseudomonas aeruginosa* (Figure 1C) but not against *S. aureus* (Figure 1A), NBE showed activity against *Staphylococcus aureus* at 25% v/v concentration (Figure 1B) but was ineffective against *Pseudomonas aeruginosa* (Figure 1D).

Effect of Neem Seed Oil and Neem bark extract on Biofilm formation of *S. aureus* and *P. aeruginosa*

Figure 2A and 2B shows the effect of NBE, NSO and NSO tween on planktonic and biofilm formation of *Staphylococcus aureus*. NSO and NSO tween both reduced biofilm formation from concentrations 25 to 50% v/v while complete killing of planktonic was found at these concentration. Complete eradication of biofilm was found with NBE at 25% v/v and 50% v/v/ while it inhibited the growth of planktonic from 3.13% v/v to 50%v/v respectively. 0.1 to 3.13% v/v of NBE induced both planktonic and biofilm. The SEM images in Figure 4A, 4B and 4C shows concentration dependent reduction of *S. aureus* biofilm at control, 25% and 50% v/v concentration of NBE respectively.

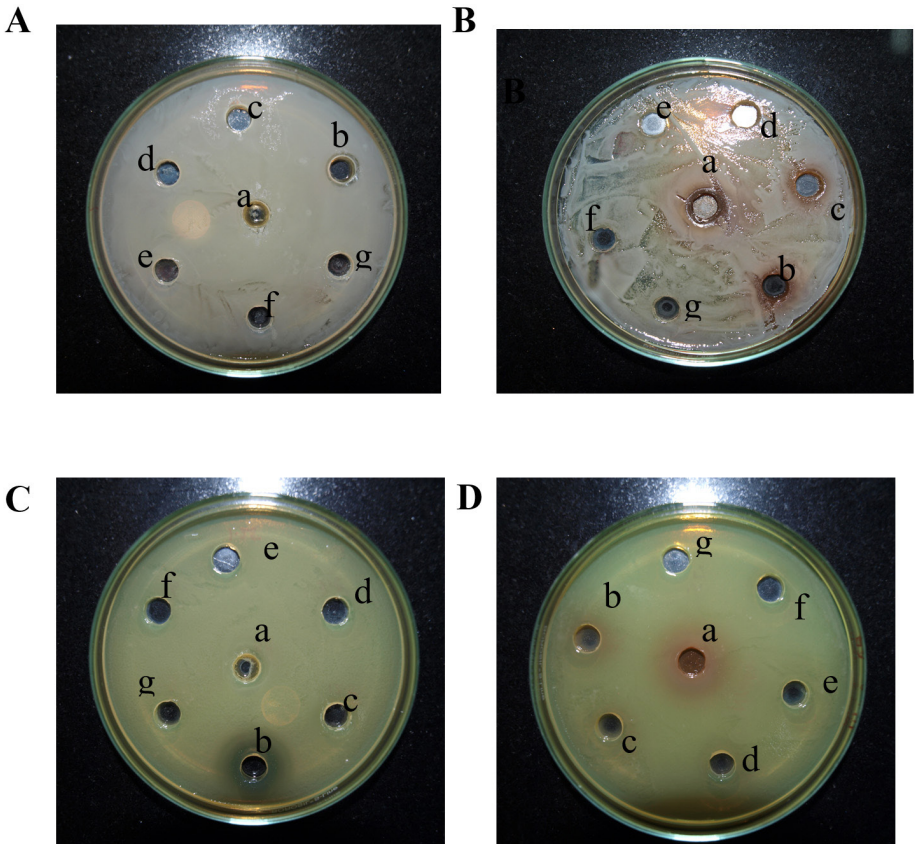

Figure 1. (A) Effect of NSO on *S. aureus*; (B) Effect of NBE on *S. aureus;* (C) Effect of NSO on *P. aeruginosa;* (D) Effect of NBE on *P. aeruginosa*
(a) 100% (b) 50% (c) 25% (d) 12.5% (e) 6.25% (f) 3.25% (g) 1.06%

Standard micro-titer biofilm formation assay was used to determine the activity of NSO against planktonic and biofilm formation in *P aeruginosa* (Figure 2C and 2D). NSO (0.1%-50% v/v) causes an inhibition in *P. aeruginosa* planktonic growth with biofilm inhibition only at concentration above 25% v/v. The presence of tween 20 on the contrary inhibited biofilm formation with no antimicrobial activity on planktonic cells at concentrations ranging from 0.1-12.5% v/v. NSO in the presence and absence of tween 20 were antimicrobial at 25% v/v concentrations for planktonic and biofilm modes of growth. NBE showed no significant effect on biofilm formation till 25% v/v concentration while induction in planktonic cell growth was also observed. Figure 2D indicates that biofilm formation was inhibited in a 24 hrs more than 12.5% v/v with MIC 90 at 50% v/v concentration. Figure 4D and 4E are SEM images of *P. aeruginosa* control and 50% v/v NSO treated biofilm. The image underline the results where biofilm has been significantly reduced.

Effect of NSO and Neem bark extract on mature biofilm of *S. aureus and P. aeruginosa*

Figure 3A and 3B shows the effect of NBE, NSO, and NSO Tween on dispersed planktonic and mature biofilm of *S.aureus*. NSO and NSO tween induced mature biofilm at 3.13 to 50% v/v concentrations, respectively, while no effect was found at 0.1 to 0.78% v/v concentrations. NBE

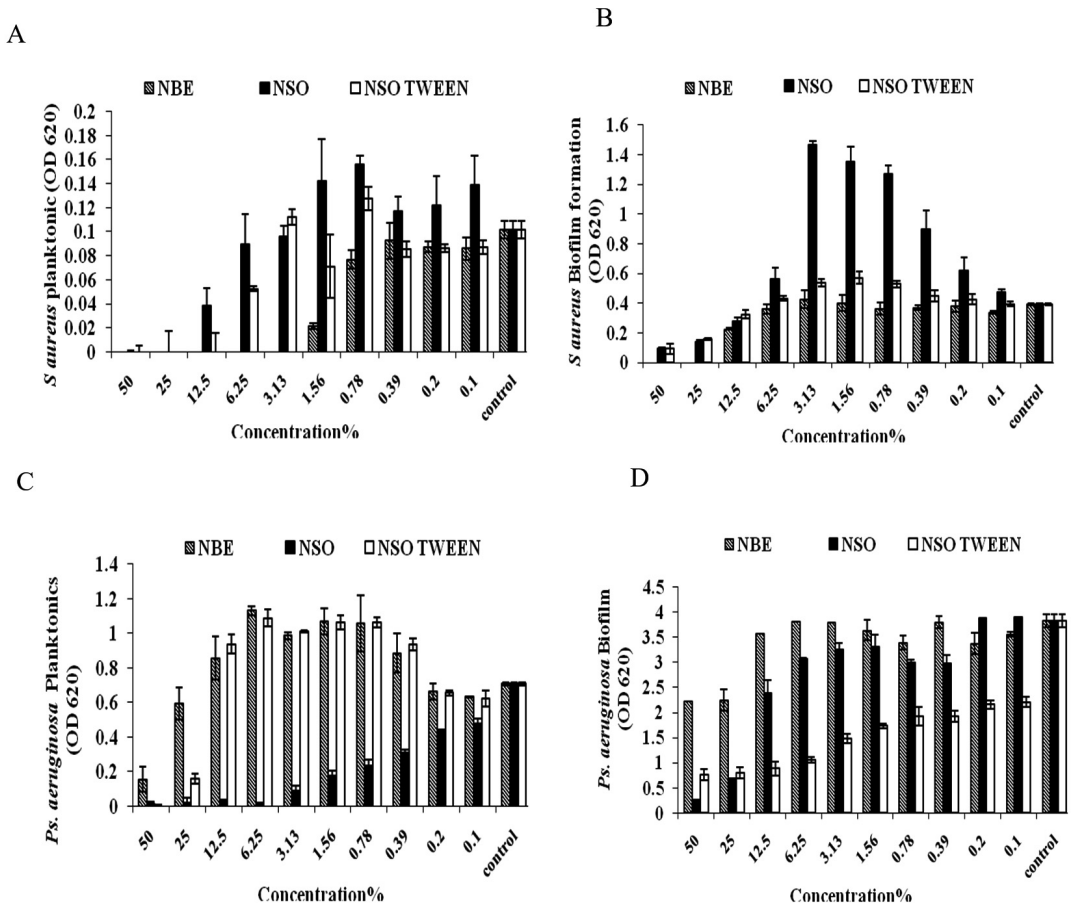

Figure 2. Effect of Neem compounds on biofilm formation assay
Planktonic (A,C) and Biofilm (B,D) for *S. aureus* and *P. aeruginosa* respectively

eradicated biofilm completely at 25 to 50% v/v while induced at moderate to 0.1 to 12.5% v/v concentrations, respectively.

Figure 3C and 3D shows the effect of NBE, NSO and NSO-tween on preformed 24 h mature *P. aeruginosa* biofilm. 24h mature biofilm is washed and treated with different concentrations of NBE, NSO and NSO-tween. Figure 3C is a spectrophotometric reading of the biofilm dispersed planktonic cells following treatment while Figure 3D represents adherent biofilm cells. A similar concentration dependant effect of NBE is seen on biofilms with no antimicrobial activity. No effect of NSO or NSO tween is seen on biofilm formation with antimicrobial activity of NSO and NSO tween only on dispersed planktonic cells at concentration above 12.5% v/v.

DISCUSSION

In this study, we compared the activity of neem bark extract (NBE) and neem seed oil (NSO) on microbial growth of model Gram positive (*S. aureus*) and Gram negative (*P. aeruginosa*) bacteria. Antibacterial activity in NSO has been attributed to phytochemicals. Tween 20 was used

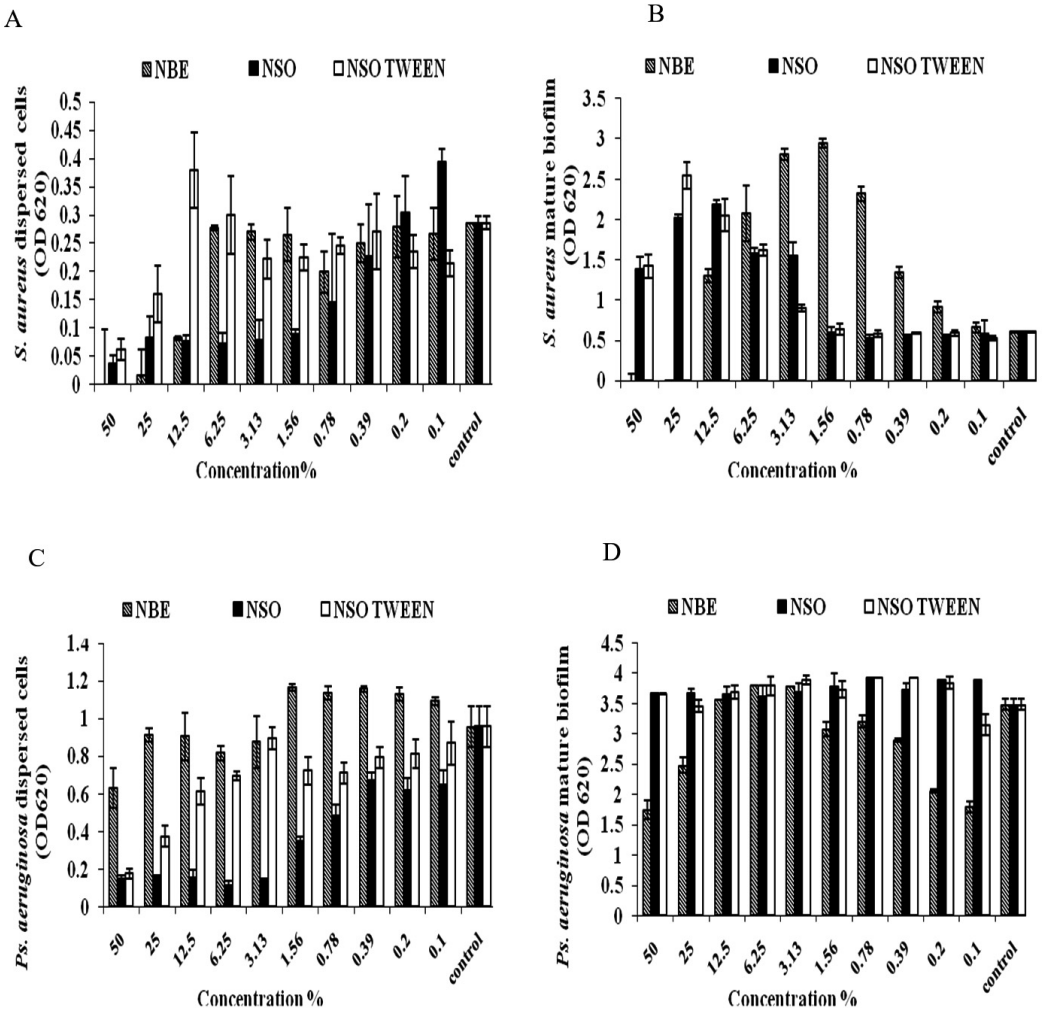

Figure 3. Effect of Neem compounds on biofilm eradication assay
Dispersed planktonic (A,C) and Mature biofilm (B,D) for *S. aureus* and *P. aeruginosa* respectively

as nonionic surfactant for NSO as its non toxic, biocompatible nature and has established use for creating nanoemulsions.[18]

In a standard zone of inhibition assay, NSO (50% v/v) was more effective against Gram negative *P. aeruginosa* while NBE (25% v/v) was effective against Gram positive *S. aureus* compared to *P. aeruginosa*. In these concentrations biofilm formation as well mature biofilms were successfully inhibited as a result of its antimicrobial activity. However, variable effects of different concentrations were found on biofilm formation. The variable effect of herbal extracts is likely determined by the growth form and metabolic age of the cells. The variable effects collated in this study are tabulated in Table.

Biofilm formation assay showed that both NSO and NBE induced the adherence of the organisms onto adherent surface. Hydrophobic nature of the oil interacts with the lipid bilayer of the outer membrane of the bacteria which could also cause increased cell permeability. Compounds with partition coefficient (Po/w) higher than 3 are reported to partition in the cell membrane.[19] Po/w of azadirachtin is reported to be 12.3 which can be affected by presence of media constituents,

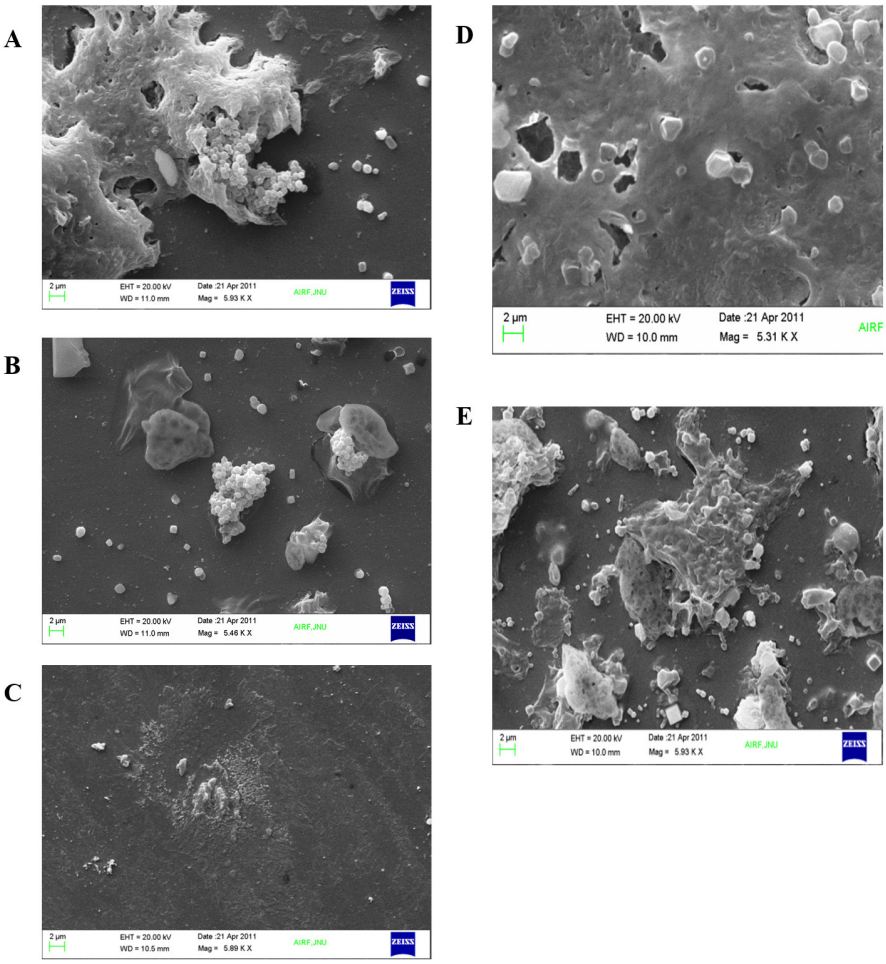

Figure 4. Scanning electron microscopy images of biofilm
A. Control (untreated *S. aureus* biofilm)
B. *S. aureus* Biofilm formation with 25% NBE
C. *S. aureus* Biofilm formation with 50% NBE
D. Control (untreated *P. aeruginosa* biofilm.)
E. *P. aeruginosa* Biofilm formation with 50% NSO

Table. (A) Effect of Neem compounds on *Ps aeruginosa*

Neem Extracts	Concentrations	Biofilm formation	Mature Biofilm
Neem bark	Very high	Inhibition ^{P,B}	Inhibition ^{DP,B}
	High	Induction ^{P}, No effect ^{B}	No effect DP, Inhibition ^{B}
	Moderate	Induction ^{P}, No effect ^{B}	Induction DP, Inhibition ^{B}
	Low	Inhibition ^{P}, No effect ^{B}	Induction DP, Inhibition ^{B}
NSO	Very high	Inhibition ^{P,B}	Inhibition DP, Induction ^{B}
	High	Inhibition ^{P,B}	Inhibition DP, Induction ^{B}
	Moderate	Inhibition ^{P,B}	Inhibition DP, Induction ^{B}
	Low	Inhibition ^{P}, No effect ^{B}	Inhibition DP, Induction ^{B}
NSO tween	Very high	Inhibition ^{P,B}	Inhibition DP, Induction ^{B}
	High	Induction ^{P}, Inhibition ^{B}	Inhibition DP, Induction ^{B}
	Moderate	Induction ^{P}, Inhibition ^{B}	Inhibition DP, Induction ^{B}
	Low	No effect ^{P}, Inhibition ^{B}	Inhibition DP, No effect ^{B}

(B). Effect of Neem compounds on *S. aureus*

Neem Extracts	Concentrations	Biofilm formation	Mature Biofilm
Neem bark	Very high	Inhibition ^{P,B}	Inhibition ^{DP,B}
	High	Inhibition ^{P,B}	Inhibition ^{DP,} Induction ^{B}
	Moderate	Inhibition P, No effect B	No effect ^{DP,} Induction ^{B}
	Low	No effect ^{P,B}	No effect ^{DP,} Induction ^{B}
NSO	Very high	Inhibition ^{P,B}	Inhibition ^{DP,} Induction ^{B}
	High	Inhibition P,Induction B	Inhibition ^{DP,} Induction ^{B}
	Moderate	Induction ^{P,B}	Inhibition ^{DP,} Induction ^{B}
	Low	Induction ^{P,B}	Induction ^{DP,} No effect ^{B}
NSO tween	Very high	Inhibition ^{P,B}	Inhibition ^{DP,} Induction ^{B}
	High	Inhibition ^{P,B}	Induction ^{DP,B}
	Moderate	Induction ^{P,B}	No effect ^{DP,B}
	Low	No effect ^{P,B}	No effect ^{DP,B}

P: Planktonic, DP, Dispersed Planktonic, B: Biofilm

cellular metabolites and presence of surfactant affecting adherence of cells for biofilm formation as well as its ability to perturb the cellular membranes.

Presence of tween inhibited biofilm formation of *Pseudomonas* while promoting its planktonic growth. Tween surfactant with NSO would result in changes in the hydrophobicity affecting the adherence ability of the bacterial cells. A similar antiadhesive activity was reported for aqueous extract of neem on *Candida albicans* strains as a consequence of changes in cell hydrophobicity.[20] At low concentrations of crude NBE, cell growth induction is likely to be caused by utilizing components as source of micronutrients which become toxic as cell concentrations increase. 0.01 g/ml of aqueous extract of neem was found to induce biofilm formation of *C. albicans* cells.[20] A bimodal effect is observed with varying concentrations of neem compounds. At high concentrations of the extract, stress may be responsible for the strong induction of biofilm growth. Even in the case of mature *P aeruginosa* biofilms, low concentrations induce biofilm dispersal (planktonic to biofilm ratio increases) with significant decrease of biofilm at 50%v/v concentrations. The effect of neem leaf extract on inhibiting *P. aeruginosa* biofilm formation by affecting exopolysaccharide formation, adhesion activity and alginate production has been reported.[11,12] Bark extract of *Azadirachta indica* has shown bactericidal activity against against *S. aureus* at higher concentration of >500 µg/mL The bark extract also showed antibacterial activity against *P. aeruginosa, E. faecalis,* and *P. mirabilis* at various concentrations.[6]

NSO may be inducing biofilm form of growth as a consequence of being utilized as a carbon source. NSO has no effect on mature *P. aeruginosa* biofilm while inhibiting dispersed cells at concentrations above 12.5% v/v. Presence of tween 20 may in turn hence protect cells from the antimicrobial activity and stress caused by NSO. While neem extracts are effective in inhibiting planktonic growth form and biofilm formation in the two organisms, its efficacy is severely impaired in its activity on preformed biofilms. The ability of the plant extract to penetrate through the exopolymeric matrix requires investigation of novel strategies. Development of bioformulations of plant extracts in a hydrophobic carrier or polysaccharide dispersive enzymes need to be explored for the effective use of natural plant products.

Methanolic extracts of neem seeds are reported to be effective against the formation and eradication of *Staphylococcus aureus* and *Vibrio cholerae* bacterial bioflm respectively at 10x higher concentrations.[4] Studies have reported effective antibiofilm activity against *S. aureus* biofilm formation *in vitro* assays using confocal laser scanning microscopy and atomic force microscopy image analysis.[21] The role of nimbolide for biofilm inhibition was also demonstrated in a methicillin resistant *S. aureus*.[5] Antimicrobial activities of methanol and ethanol extracts of the tested neem oil extracts have been attributed to the presence of many secondary plant metabolites such as isoprenoids (limonoid structures) and non-isoprenoids (e.g., tannins).[10] Hence, there maybe several explanations for the antibiofilm activity of NSO tween concentrations which may include (a) increased permeability of NSO tween through the biofilm matrix (b) decreasing adherence of cells for biofilm formation (c) cellular membrane perturabation.

Although the mechanisms by which concentration dependent effect of neem seed oil and its extract affect microbial growth are not well understood, the current data suggest that pleiotropic effects of the phytochemicals on inhibiting microbial growth or inducing biofilm mode of growth of model Gram positive and Gram negative organisms must be taken into consideration while studying their antimicrobial effects. High concentrations of neem products are known to cause eukaryotic cellular toxicity and hence safe concentrations of the compounds must be considered before their usage in clinical settings.[22]

This study has its limitations as results come from a combination from active ingredients in NSO and NBE, and the use of purified extracts will provide defined information regarding Neem antibiofilm activity. In future, analysis of nanoemulsion based antibiofilm activity of NSO can be performed for the development of neem based antibiofilm products.

CONCLUSION

This work demonstrates the importance of concentration dependent killing properties of Neem derived phytochemicals wherein one can attain maximum anti-bacterial and anti-biofilm concentration with the most optimal concentrations. Since biofilms are induced in response to stress, it is important to determine the concentration of anti-microbial compounds that are effective in penetrating the biofilm exopolymeric substances resulting in cellular lysis and not inducing signal transduction pathways providing biofilm inducing signals. The study highlights the importance of understanding the scientific mechanisms of traditional plant bioformulations to obtain maximum antimicrobial effects with reduced adverse consequences. Neem and its compounds represent high commercial value and are an significant candidate for alternative and integrated drug therapy regimens.

ACKNOWLEDGMENTS

The authors would like to thank Department of Microbiology, CSJM University Kanpur for providing research facilities.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

REFERENCES

1. Islas JF, Acosta E, G-Buentello Z et al., An overview of Neem (Azadirachta indica) and its potential impact on health. *J. Funct. Foods*, 2020; 74, 104171. doi: 10.1016/j.jff.2020.104171
2. Chaudhary MF, Ashraf A, Waseem M, et al. Neem oil. In Inamuddin, R. Boddula, & A. M. Asiri (Eds.), Green Sustainable Process for Chemical and Environmental Engineering and Science. 2021:57–73. Elsevier. doi: 10.1016/B978-0-12-821886-0.00008-7
3. Ghosh S, Mali SN, Bhowmick DN, Pratap AP. Neem oil as natural pesticide: Pseudo ternary diagram and computational study. *J.Indian Chem. Soc.* 2021:98(7), 100088. doi: 10.1016/j.jics.2021.100088
4. Guchhait KC, Manna T, Barai M et al. Antibiofilm and anticancer activities of unripe and ripe Azadirachta indica (neem) seed extracts. *BMC Complement Med Ther.* 2022; 22(42). doi: 10.1186/s12906-022-03513-4
5. Sarkar P, Acharyya S, Banerjee A et al. Intracellular,biofilm-inhibitory and membrane damaging activities of nimbolide isolated from Azadirachta indica A. Juss (Meliaceae) against methicillin-resistant Staphylococcus aureus. *J.Med. Microbiol.* 2016; 65(10), 1205–1214. doi: 10.1099/jmm.0.000343
6. Singaravelu S, Sankarapillai J, Sasidharn CA, Sinha P. Effect of Azadirachta indica Crude Bark Extracts Concentrations against Gram-Positive and Gram-Negative Bacterial Pathogens. J Pharm Bioallied Sci. 2019;11(1):33-37. doi: 10.4103/JPBS.JPBS_150_18
7. Qin S, Xiao W, Zhou C et al. Pseudomonas aeruginosa: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. In *Signal Transduction and Targeted Therapy* 2022; (7):1 Springer Nature. doi: 10.1038/s41392-022-01056-1
8. Howden BP, Giulieri SG, Wong T, et al. Staphylococcus aureus host interactions and adaptation. *Nat. Rev. Microbiol.*.2023; 21: 380-395. doi: 10.1039/s41579-023-00852y
9. Hoiby N, Bjarnsholt T, Givskov M et al.. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents*. 2010;35(4):322-332. doi: 10.1016/j.ijantimicag.2009.12.011
10. Wylie MR & Merrell DS. The Antimicrobial Potential of the Neem Tree Azadirachta indica. *Front. Pharmacol*, 2022; 13: 891535. doi: 10.3389/fphar.2022.891535
11. Harjai K, Bala A, Gupta RK, Sharma R. Leaf extract of Azadirachta indica (neem): A potential antibiofilm agent for Pseudomonas aeruginosa. *Pathog.Dis.* 2013; 69(1) 62–65. doi: 10.1111/2049-632X.12050
12. Kaverimanian V and Heuertz RM. Effects of Neem Extracts on Formed Biofilm of Pseudomonas aeruginosa. *The FASEB Journal*, 2020; *34*(S1), 1. doi: 10.1096/fasebj.2020.34.s1.05737
13. Høiby N, Bjarnsholt T, Givskov M. et al. Antibiotic resistance of bacterial biofilms. *Int. J.Antimicrob. Agents*, 2020; 35(4): 322-332. doi: 10.1016/j.ijantimicag.2009.12.011
14. Kumar S, Mulchandani V, Das Sarma JS. Methanolic neem (Azadirachta indica) stem bark extract induces cell cycle arrest, apoptosis and inhibits the migration of cervical cancer cells in vitro. *BMC Complement Med Ther*. 2022;22(1):239. doi: 10.1186/s12906-022-03718-7
15. Humphries RM, Ambler J, Mitchell SL, et al. CLSI Methods Development and Standardization Working Group of the Subcommittee on Antimicrobial Susceptibility Testing. CLSI Methods Development and Standardization Working Group Best Practices for Evaluation of Antimicrobial Susceptibility Tests. *J Clin. Microbiol.* 2018; *56*(4). doi: 10.1128/JCM.01934-17
16. Sagar, SS, Kumar R, Kaistha, SD. Inhibition of different stages of biofilm of Pseudomonas aeruginosa PA 01 by isolated bacteriophage P2. *Asian J Pharm Clin Res*. 2015; *8*(2): 296-299. https://journals.innovareacademics.in/index.php/ajpcr/article/view/4457.
17. Sagar, SS, Kumar R, Kaistha SD. Efficacy of Phage and Ciprofloxacin Co-therapy on the Formation and Eradication of Pseudomonas aeruginosa Biofilms. *Arab J Sci Eng.* 2017; *42*(1), 95–103. doi: 10.1007/s13369-016-2194-3
18. Azeem A, Rizwan M, Ahmad FJ, et al. Nanoemulsion components screening and selection: a technical note. *AAPS Pharm Sci Tech*. 2009;10(1):69-76. doi: 10.1208/s12249-008-9178-x
19. Zocher F, van der Spoel D, Pohl P, Hub JS. Local partition coefficients govern solute permeability of cholesterol-containing membranes. *Biophys J*. 2013;105(12):2760-2770. doi: 10.1016/j.bpj.2013.11.003
20. Polaquini SRB, Svidzinski, TIE, Kemmelmeier C et al. Effect of aqueous extract from Neem (Azadirachta indica A. Juss) on hydrophobicity, biofilm formation and adhesion in composite resin by Candida albicans. *Arch.Oral Biol.* 2006; 51(6), 482–490. doi: 10.1016/j.archoralbio.2005.11.007
21. Quelemes PV, Perfeito MLG, Guimarães, MA et al., Effect of neem (Azadirachta indica A. Juss) leaf extract on resistant Staphylococcus aureus biofilm formation and Schistosoma mansoni worms. *J.Ethnopharmacol.* 2015; 175, 287–294. doi: 10.1016/J.JEP.2015.09.026
22. Braga TM, Rocha L, Chung TY, et al. Azadirachta indica A. Juss. In Vivo Toxicity-An Updated Review. *Mol.*.2021;26(2):252. doi: 10.3390/molecules26020252